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cDNAs of aminopeptidase-like protein genes from *Plodia interpunctella* strains with different susceptibilities to *Bacillus thuringiensis* toxins

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Abstract

Aminopeptidase N has been reported to be a *Bacillus thuringiensis* (Bt) Cry1A toxin-binding protein in several lepidopteran insects. cDNAs of aminopeptidase-like proteins from both Bt-susceptible RC688^s and Bt-resistant HD198^r strains of the Indianmeal moth, *Plodia interpunctella*, were cloned and sequenced. They contain 3345 and 3358 nucleotides, respectively, and each has a 3048 bp open reading frame that encodes 1016 amino acids. Putative protein sequences include 10 potential glycosylation sites and a zinc metal binding site motif of HE*XX*H, which is typical of the active site of zinc-dependent metallopeptidases. Sequence analysis indicated that the deduced protein sequences are most similar to an aminopeptidase from *Heliothis virescens* with 62% sequence identity and highly similar to three other lepidopteran aminopeptidases from *Plutella xylostella, Manduca sexta, Bombyx mori* with sequence identities of 51–52%. Four nucleotide differences were observed in the open reading frames that translated into two amino acid differences in the putative protein sequences. Polymerase chain reaction (PCR) confirmed an aminopeptidase gene coding difference between RC688^s and HD198^r strains of *P. interpunctella* in the PCR amplification of a specific allele (PASA) using preferential primers designed from a single base substitution. The gene mutation for Asp^{185}→Glu^{185} was also confirmed in two additional Bt-resistant *P. interpunctella* strains. This mutation is located within a region homologous to the conserved Cry1Aa toxin binding regions from *Bombyx mori* and *Plutella xylostella*. The aminopeptidase-like mRNA expression levels in the Bt-resistant strain were slightly higher than those in the Bt-susceptible strain. The sequences reported in this paper have been deposited in the GenBank database (accession numbers AF034483 for susceptible strain RC688^s and AF034484 for resistant strain HD198^r).

Keywords: Aminopeptidase; PASA; cDNA; Gene; Amino acid sequence; Indianmeal moth; *Plodia interpunctella*; *Bacillus thuringiensis*

1. Introduction

The Indianmeal moth, *Plodia interpunctella*, is one of the economically important lepidopteran pests of stored grain products. Control of this insect using environmentally safe biopesticides, such as insecticidal crystal proteins (ICPs) produced by *Bacillus thuringiensis* (Bt), has encountered difficulty because of the development of resistance to Bt toxins (McGaughey, 1985; McGaughey and Beeman, 1988; McGaughey and Johnson, 1992).

ICPs are known as δ-endotoxins or crystal (Cry) gene products with species-specific toxicity for the larvae of a number of agronomic pests and disease vectors. After ingestion by a susceptible insect, protoxins of lepidopteran-active ICPs are dissolved in the alkaline environment of the insect midgut and proteolytically activated by midgut proteinases resulting in 60–65 kDa toxins. The activated toxins bind to receptors on the surface of midgut epithelial cells, which results in a change of permeability of the gut membranes, lysis of the midgut epithelial cells, and death of the insect (English and Slatin, 1992; Bauer, 1995; Cannon, 1996).

Cry1A toxins are a major group of δ-endotoxins active against a variety of lepidopteran larvae. In most insects, the toxicity of Bt endotoxins is correlated with binding to receptors in the epithelial brush border of the midgut (Van Rie et al., 1990; Estada and Ferre, 1994). The inter-

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action of these receptors with Bt toxins is a key determinant for toxin specificity (Hofmann et al., 1988; Van Rie et al., 1990). Aminopeptidase-like proteins have been documented to be major binding proteins for Cry1A toxins in several species (Sangadala et al., 1994; Garczynski and Adang, 1995; Gill et al., 1995; Knight et al., 1995; Masson et al., 1995; Valaitis et al., 1995; Luo et al., 1997; Chang et al., 1999). Recently, a Cry1Aa binding region of aminopeptidase-N (APN) from *Bombyx mori* and *Plutella xylostella* was found near the N-terminus (Nakanishi et al., 1999). Bt-resistance in many field populations and laboratory strains is caused by a change in the midgut membrane receptor for Bt toxins, which reduces binding affinity or abolishes binding to the receptor molecule (reviewed in McGaughey and Oppert, 1999; Schnepf et al., 1998). Therefore, we examined differences in putative APN-like receptor molecules in Bt-susceptible and resistant strains of *P. interpunctella*.

cDNAs for aminopeptidase genes from several lepidopteran insects have been cloned and sequenced (Gill et al., 1995; Knight et al., 1995; Chang et al., 1999), but sequence information has been collected only from Bt-susceptible strains. To determine whether molecular differences are present in the genes encoding aminopeptidases from resistant insects, we examined cDNAs, mRNA expression levels, and genomic restriction sites for aminopeptidase-like protein genes from both Bt-susceptible and Bt-resistant strains of *P. interpunctella*.

2. Materials and methods

2.1. Insect cultures

P. interpunctella strain, RC688^{s} (Bt-susceptible), was collected from farm-stored grain in Riley County, Kansas, and maintained on a cracked wheat diet described by McGaughey and Beeman (1988). A Bt-resistant strain, HD198^{r}, was selected from RC688^{s} using Bt subsp. *entomocidus* HD-198 incorporated into diet (McGaughey and Johnson, 1992).

2.2. mRNA purification and cDNA library construction

Total RNA was extracted with guanidine thiocyanate denaturing solution and precipitated with isopropanol (Titus, 1991). The poly(A) RNA was isolated from the total RNA by chromatography on an oligo(dT)-cellulose column (Gibco BRL Life-Technologies, Gaithersburg, MD). Double stranded cDNA was synthesized using 5 μg of poly(A) RNA as a template (ZAP-cDNA synthesis kit, Stratagene, La Jolla, CA), directionally cloned into a UniZAP XR vector phage (Stratagene), and packaged using the ZAP-cDNA Gigapack II Gold cloning system (Stratagene). Approximately 2 and 1.7 million recombinants were obtained for RC688^{s} and HD198^{r} cDNA libraries, respectively.

2.3. Development of probe and cDNA library screening

Lambda DNA of an amplified RC688^{s} cDNA library was prepared using phage precipitation and phenol/chloroform extraction procedures after RNase A and DNase I digestions. Polymerase chain reaction (PCR) was carried out with two degenerate primers, 5′-GGNGCNATGGARAAYTGGGG-3′ as a forward primer and 5′-GCRAANCCYTCRTTNARCCA-3′ as a reverse primer, designed from two highly conserved regions (GAMENWG and WLNEGFA) of an aminopeptidase from *Heliothis virescens* (Fab.) (Gill et al., 1995). PCR-amplified DNA fragments (~200 bp) were cloned into a pGEM-T vector, and a sequence of the fragment was obtained using silver staining protocols (Promega, Madison, WI). The cDNA libraries of both strains were screened using an α-^{32}P-dCTP-labeled probe (Amersham, Arlington Heights, IL). At least two clones from each library were sequenced in both directions. The BLASTX non-redundant protocol was used to perform sequence similarity searching and retrieval of homologous sequences from GenBank of the National Center for Biotechnology Information. The Wisconsin Sequence Analysis Package GCG Unix version 9.0 (Genetics Computer Group, Madison, WI) and sequence analysis tools of the SWISS-PROT Internet server were used to process data of deduced protein sequences.

2.4. Northern analysis of aminopeptidase mRNA expression

Aminopeptidase-like mRNA expression in fourth instar larvae of the two strains of *P.interpunctella* was analyzed by Northern blotting (Ausubel et al., 1994). Three μg of mRNA from each strain was subjected to 1% agarose/formaldehyde gel electrophoresis and transferred to a nylon membrane (MSI, Westboro, MA). PCR-amplified aminopeptidase-like cDNA fragments were labeled with α-^{32}P-dCTP to probe the target aminopeptidase mRNA. To evaluate loading quantity, the aminopeptidase-like cDNA probe was removed from the nylon membrane using 0.5% boiling SDS solution after the film was processed. The membrane was rehybridized with α-^{32}P-labeled ribosomal protein S3 cDNA from the tobacco hornworm, *Manduca sexta* (L.), as an internal standard, which is a highly conserved sequence among bacteria, yeast, vertebrates, and invertebrates (Jiang et al., 1996).

2.5. PCR amplification of specific allele (PASA)

After full cDNA sequences were obtained from both susceptible and resistant libraries, differences of four

nucleotides in the open reading frames were found between the two strains. To confirm that the structural change was not an artifact of cloning, the PASA technique was used to amplify genomic DNA extracted from individual fourth instar larvae. This technique is a general method for detecting known single-base changes as well as small deletions and insertions. Oligonucleotide primers are designed and used to preferentially amplify one allele over another (Sommer et al., 1992). Specific amplification can be obtained if the oligonucleotide matches the desired allele but mismatches the other allele at the 3′ end of the oligonucleotide, thus preventing efficient 3′ elongation by *Taq* polymerase. To perform PASA amplification of genomic DNA from the Indianmeal moth, two pairs of primers were designed from a single base substitution at nucleotide 555 of the cDNA sequences (Fig. 1). At the point of substitution, a cytosine occurs in the cDNA from the Bt-susceptible strain, whereas an adenine is found in the cDNA from the Bt-resistant strain. A forward primer AF1 for both strains and a reverse primer SARG with a guanine at the 3′ terminal create a C/G match for the susceptible strain (designated as susceptible primers, see sequence in Fig. 1) and an A/G mismatch for the resistant strain. This mismatch reduces the PCR yield dramatically (Kwok et al., 1990). With a reverse primer AR6 common to both strains, a forward primer RAFA with adenine at the 3′ terminal creates a G/A mismatch to the susceptible strain and a perfect match to the resistant strain (designated as resistant primers). PCR reactions contained 10 mM Tris-HCl, pH 9, 1.5 mM $MgCl_2$, 1 μM of each primer, 50 mM KCl, 0.1 mM of each dNTP, 0.05 unit/μl of Taq DNA polymerase, and 20 ng of DNA template. DNA was initially denatured for 3 min at 94°C, and the PCR amplification was conducted with 35 cycles, including 30 s of denaturing at 94°C, 30 s of annealing at 59°C, and a 1 min extension time at 72°C. PCR products were separated by electrophoresis on a 1% agarose gel, stained with 0.5 μg/ml ethidium bromide, and photographed under UV light.

To examine whether the gene mutation of $Asp^{185} \rightarrow Glu^{185}$ occurred in other Bt-resistant Indianmeal moths, ten larvae of *P. interpunctella* strains $HD112^r$ and $HD133^r$ were obtained, and larval genomic DNA was individually isolated. PASA amplifications were conducted using susceptible and resistant primer sets under the same PCR conditions as stated above.

3. Results and discussion

3.1. Sequence differences in cDNAs from Bt-susceptible and resistant strains

The cDNA libraries of $RC688^r$ and $HD198^r$ were screened with the aminopeptidase-like cDNA probe. Several clones were identified from each library and two clones were selected from each for sequencing in both directions. The sequence data showed that the cDNAs cloned from the same library were identical. A cDNA of 3345 bp was obtained from the Bt-susceptible strain $RC688^s$ and a cDNA of 3358 bp was obtained from the Bt-resistant strain $HD198^r$. Both cDNAs contained 3048 bp open reading frames with the start codon ATG at positions 1–3 and the termination codon TAA at positions 3349–3351 (Fig. 1). The cDNAs encoded 1016 amino acid residues in predicted immature forms of aminopeptidase-like proteins. A polyadenylation signal, AATAA, was located at positions 3277–3282 in the $RC688^s$ cDNA and at 3266–3271 in $HD198^r$ cDNA. Alignment of the two cDNA sequences showed four nucleotide differences in the open reading frames accounting for a 0.13% sequence difference between the two strains. At nucleotide positions 318, 555, 2288, and 2571, nucleotides A, C, T, and C in Bt-susceptible strain cDNA were replaced by C, A, C, and T, respectively, in the Bt-resistant strain cDNA (Fig. 1). At the non-translatable 3′-end region (positions 3153–3163), a deletion of 11 nucleotides occurred in the Bt-resistant strain cDNA relative to the Bt-susceptible strain cDNA.

3.2. Putative amino acid sequences and similarity to aminopeptidase Ns

The putative sequences of the preaminopeptidase-like proteins contain 1016 amino acid residues with a theoretical molecular mass of 115 kDa. An 18-amino-acid signal peptide, MAAMKWFLLGVLCVSAQA, was predicted using SignalP Version 1.1 software (WWW Server) and a 23-amino-acid transmembrane region, DSAVTSALSVVAIAVAAIVNLAL, at C-terminal positions 994–1016 using SWISS-PROT Internet server PSORT protocol software. Motif H^{380} E^{381} XXH^{384} for the aminopeptidase N zinc iron binding site, which is part of a typical catalytic active site for the majority of zinc-dependent metallopeptidases, was predicted using GCG protein analysis Motifs protocol software (Figs. 1 and 2). The putative protein sequences include 10 potential glycosylation sites at amino acid residues 116, 154, 188, 280, 364, 592, 610, 645, 865 and 886.

A search of the GenBank database and use of GCG GAP comparison software revealed that the amino acid sequence predicted from *P. interpunctella* cDNA is most similar to the tobacco budworm (*H. virescens*) aminopeptidase N, a Bt Cry1Ac binding protein (Gill et al., 1995), with 62% sequence identity. Other highly similar proteins include two putative Cry1Ac-binding aminopeptidases in the diamondback moth, *Plutella xylostella* (L.) (Chang et al., 1999; 52% identity) and tobacco hornworm, *M. sexta* (Knight et al., 1995; 51% identity), as well as one putative Cry1 Aa-binding aminopeptidase N in the silk worm, *Bombyx mori* (Yaoi and Sato, 1998;

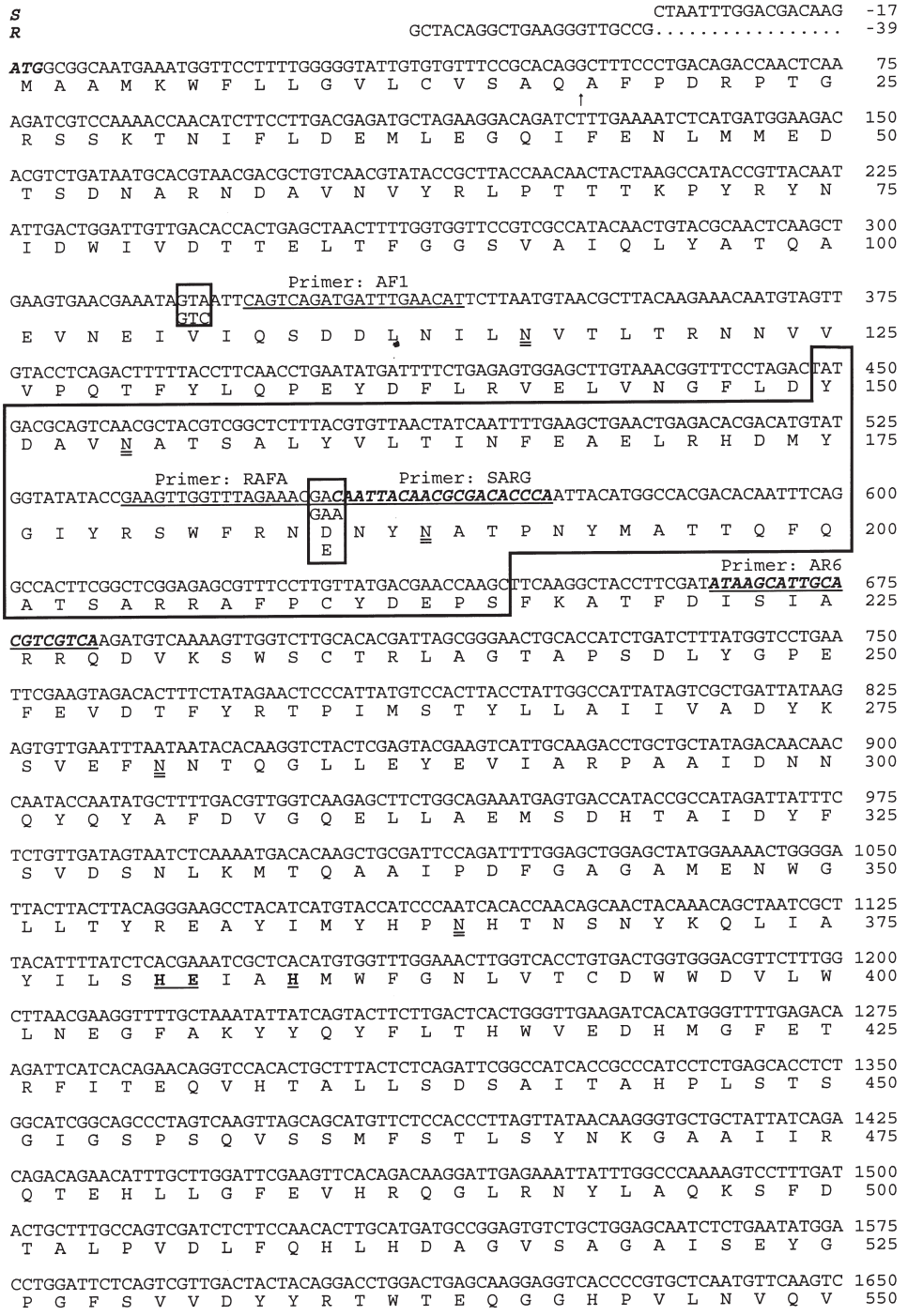

Fig. 1. Nucleotide and deduced amino acid sequences of aminopeptidase-like cDNA isolated from *P. interpunctella*. ***S***=clone from susceptible strain RC688^{s}; ***R***=clone from resistant strain HD198^{r}. Identical nucleotides between susceptible and resistant strains are indicated by dots at both 5′ and 3′ non-translatable regions; different nucleotides and resulted different amino acids between susceptible and resistant strains are boxed; nucleotide deletion is indicated by "-"; ***ATG***=start codon; ***TAA***=termination codon; ***AATAAA***=polyadenylation signal; ↑=predicted signal peptide cleavage site; potential glycosylation sites are double underlined; bold and underlined letters ***His***^{380}, ***Glu***^{381}, and ***His***^{384} represent motif residues of zinc binding active site. Forward primer sequences for PASA detection are underlined, and the sequences corresponding to reverse primers are indicated by underlined italic letters. Large boxed-in region is the proposed Cry1Aa binding region (Nakanishi et al., 1999).

51% identity). Other homologous sequences include aminopeptidases from swine, *Sus scrofa* (Delmas et al., 1994; 47% identity), human, *Homo sapiens* (Olsen et al., 1988; 45% identity), the Norway rat, *Rattus norvegicus* (Malfroy et al., 1989; 45% identity), and chicken, *Gallus gallus* (Midorikawa et al., 1998; 45% identity).

The predicted amino acid sequence encoded by the susceptible strain cDNA was aligned with 4 lepidopteran

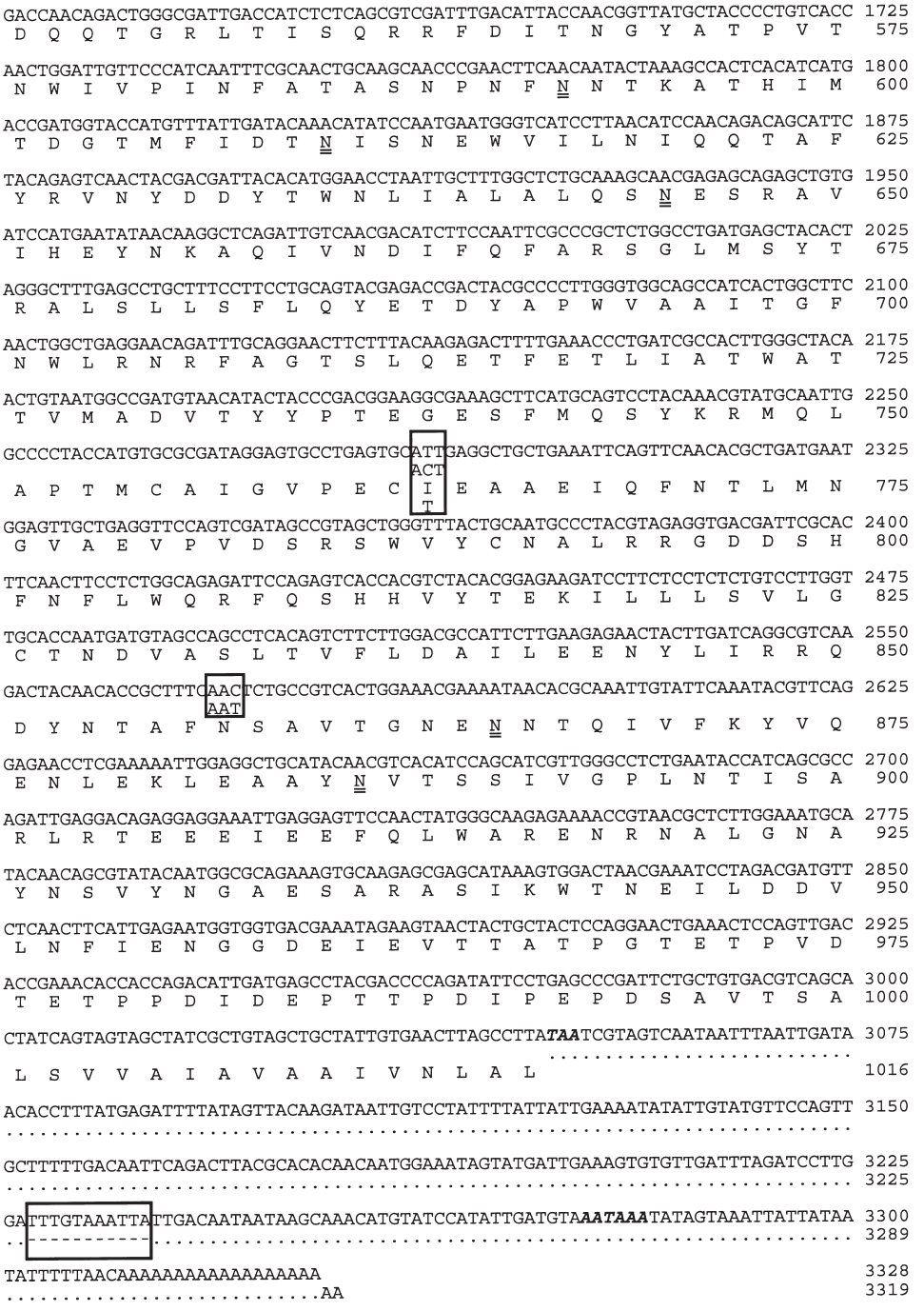

Fig. 1. (*continued*)

and 4 vertebrate aminopeptidases using the GCG Pileup program (Fig. 2, gap weight=2, gap length weight=1). This sequence contains all of the conserved residues in the putative active site, His^{380}, Glu^{381} and His^{384}, which form the catalytic zinc ion binding site. Glu^{403} is also conserved in all nine species, which also was described as a zinc binding site in the four vertebrates and in *H. virescens* (Fig. 2). In all three of the mammalian aminopeptidases, amino acids 2–8 were defined as a cytoplasmic region, which have low similarity with insect APNs, and amino acids 9–32 as a transmembrane region. In *M. sexta* and *H. virescens*, the last 22 amino acids comprise a lipid binding region, which is absent in the mature forms (Gill et al., 1995; Knight et al., 1995) and not present in mammalian APNs.

3.3. Amino acid sequence differences

Although four nucleotide differences occur in the cDNA open reading frames between the Bt-susceptible

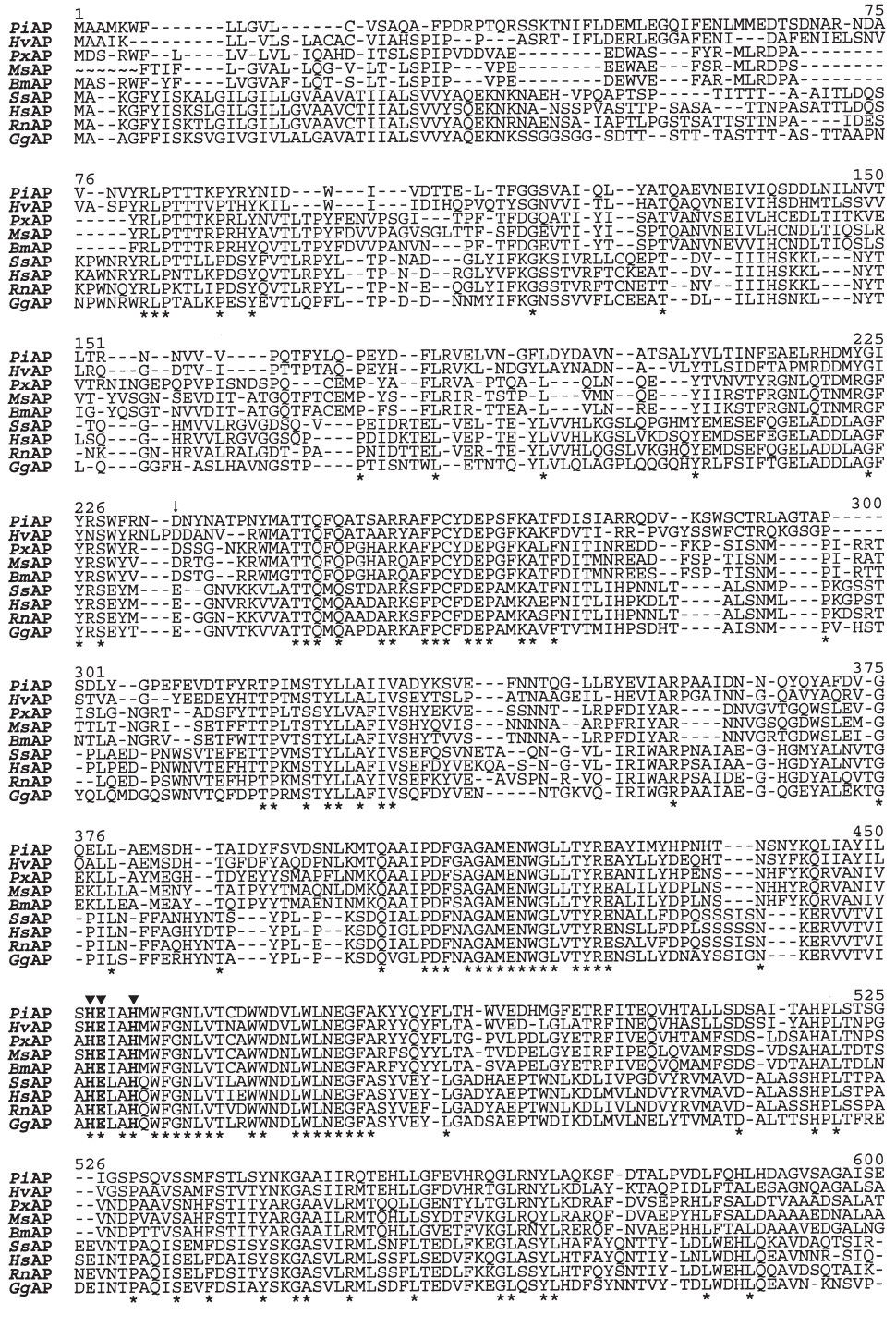

Fig. 2. Predicted amino acid sequences of aminopeptidase-like proteins from susceptible *P. interpunctella* and alignment with aminopeptidases from four other insect species and four vertebrate species. ***Pi*Ap**=aminopeptidase-like sequence of *P. interpunctella*; ***Hv*AP**= aminopeptidase N of *H. virescens* (Swiss Prot: Q11000; Gill et al., 1995); ***Px*AP**= aminopeptidase N of *P. xylostella* (GenBank: AF020389; Chang et al., 1999); ***Ms*AP**= aminopeptidase N of *M. sexta* (Swiss Prot: Q11001; Knight et al., 1995); ***Bm*AP**= aminopeptidase N of *B. mori* (GenBank: AF084257; Yaoi and Sato, 1998); ***Ss*AP**= aminopeptidase N of *S. scrofa* (Swiss Prot: P15145; Delmas et al., 1994); ***Hs*AP**= aminopeptidase N of *H. sapiens* (Swiss Prot: P15144; Olsen et al., 1988); ***Rn*AP**= aminopeptidase M of *R. norvegicus* (Swiss Prot: P15684, Malfroy et al., 1989); ***Gg*AP**=aminopeptidase N of *G. gallus* (DDBJ: D87992; Midorikawa et al., 1998). Conserved and functionally important residues (zinc binding site) are indicated by bold letters and solid triangle (▼) on the top of sequences. Identical residues among all eight sequences are indicated with star (*) at the bottom of sequences. Residues that are different between Bt-susceptible and -resistant APN sequences are marked with arrows (↓) on the top of sequences. Hyphens (- - -) represent sequence alignment gaps.

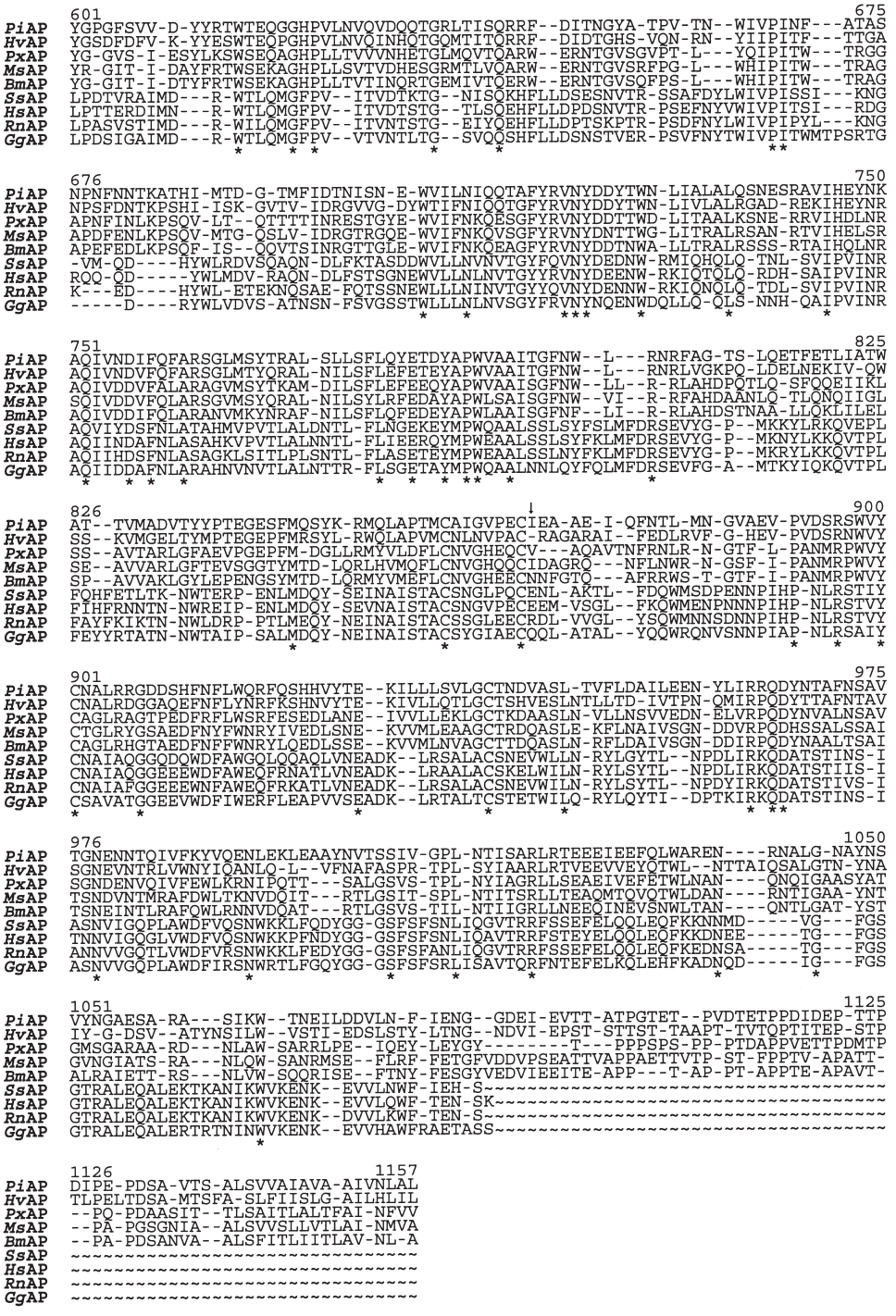

Fig. 2. (*continued*)

and Bt-resistant strains, two of the nucleotide changes are silent and the other two result in amino acid changes in the putative protein sequence of the preaminopeptidase-like enzyme. In the Bt-susceptible strain, codon GAC (nucleotides 553–555) encodes an aspartic acid which is conserved in all five insect aminopeptidases (Asp[185], Figs. 1 and 2). In the Bt-resistant strain, codon GAA encodes a glutamic acid which is conserved in all four vertebrate aminopeptidases (Glu[185], Figs. 1 and 2). This difference was located in a region corresponding to the Cry1Aa binding region in *B. mori* and *P. xylostella* (Nakanishi et al., 1999), and therefore could result in a difference in toxin affinity. Because Cry toxins bind selectively to only some APNs, it has been speculated that small differences in amino acid sequences in the toxin binding region may have a significant effect on toxin binding and susceptibility (Nakanishi et al., 1999). At another location, codon ATT encodes an aliphatic

residue isoleucine (Ile[763], Fig. 1) in the Bt-susceptible strain and codon ACT encodes the hydroxyl-containing residue threonine (Thr[763], Fig. 1) in the Bt-resistant strain. In addition to the threonine residue providing a potentially new phosphorylation site, it is adjacent to a conserved cysteine residue, which could disrupt the tertiary structure if the cysteine residue participates in a disulfide linkage. Whether a functional difference results from the gene modifications that lead to amino acid substitutions in the two *P. interpunctella* strains is unknown. It was not possible at this time to predict any structural differences in the two aminopeptidases because the insect aminopeptidase sequences did not align with other aminopeptidase sequences whose 3-D structures are available in the Protein Data Bank.

3.4. Aminopeptidase mRNA expression

To examine whether aminopeptidase-like gene expression is the same in the two *P. interpunctella* strains, northern blots containing mRNAs from RC688[s] and HD198[r] were hybridized with a PCR-generated probe. The aminopeptidase-like cDNA probe hybridized to mRNAs and yielded fragment sizes of ~3.3 kb (Fig. 3), indicating that the cDNAs cloned from susceptible and resistant strain cDNA libraries were full length cDNAs. The aminopeptidase-like mRNA expression level of the Bt-resistant strain was only slightly higher than that of the Bt-susceptible strain based on autoradiogram intensity (Fig. 3A). The different intensities may have resulted from degradation of mRNA or from a small mRNA fragment that is homologous to the aminopeptidase mRNA. However, hybridization to a loading control protein (S3) mRNA was similar between the two strains and no degradation of mRNA in the Bt-resistant strain were evident (Fig. 3B).

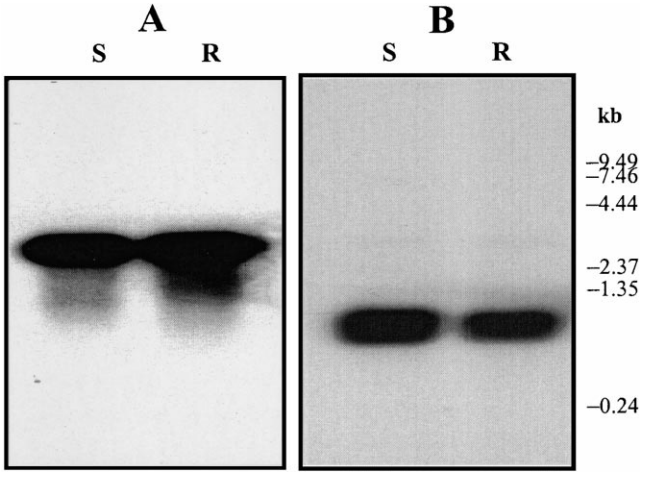

Fig. 3. Comparison of aminopeptidase-like mRNA expression levels in two *P. interpunctella* strains. S=Bt susceptible strain RC688[s]; R=Bt resistant strain HD198[r]. (A) Three μg of mRNA per lane from each strain was subjected to 1% agarose/formaldehyde gel electrophoresis and transferred to a nylon membrane. mRNA was hybridized with aminopeptidase cDNA probe labeled with ^{32}P-dCTP. (B) Same nylon membrane for panel A, and RNA was hybridized with a ribosomal protein S3 for an mRNA loading control.

3.5. PASA confirmation of aminopeptidase gene difference

To investigate whether the base substitutions located in the genome are the result of transcriptional or post-transcriptional modification or are artifacts of cloning, the PASA technique was used to amplify genomic DNA extracted individually from ten Bt-susceptible (RC688[s] and ten resistant (HD198[r]) larvae. PASA amplification was conducted using susceptible and resistant primers. Fragments of ~250 bp were amplified only from the Bt-susceptible larvae and not from the Bt-resistant larvae when susceptible primers were used (Fig. 4A). Similar lengths of cDNAs (253 bp) and PASA products indicated that no intron was present in the genomic DNA between the binding sites of the two susceptible primers. When the resistant primers were used, ~600 bp fragments were amplified from the Bt-resistant larvae but not the Bt-susceptible larvae (Fig. 4B). Fragments of the PASA products were longer than the cDNA (148 bp) between the two Bt-resistant primers, indicating that introns were located between the two priming sites on the genomic DNA. The results of PASA analysis confirmed that the aminopeptidase genes are different in Bt-susceptible and -resistant strains of *P. interpunctella* and indicated genomic organizational differences between the two strains.

To test for a gene structure modification that is associated with Bt resistance in *P. interpunctella*, PASA amplifications were extended to include two more Bt-

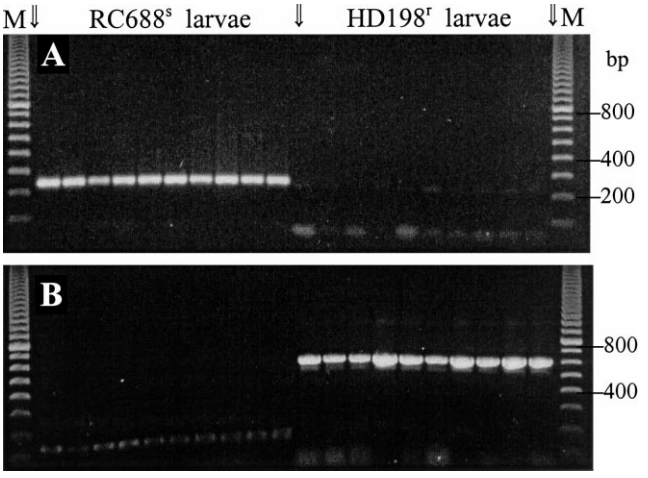

Fig. 4. PASA amplification of individual larval DNA of both Bt susceptible RC688[s] and Bt resistant HD198[r] insects. Ten individuals from each strain were used for each pair of primers. (A) PASA amplification using susceptible primers. (B) PASA amplification using resistant primers. M=100 bp DNA ladder from Pharmacia.

resistant strains, HD112^r and HD133^r, which are resistant to isolates HD-112 and HD-133, respectively, of Bt subsp *aizawai*. These two strains have a broad spectrum of resistance to toxins CryIAb, CryIAc, and CryIC (McGaughey and Johnson, 1994). The data showed that ~600 bp fragments were amplified from both strain HD112^r (Fig. 5A) and HD133^r (Fig. 5B) by using resistant primers. No ~250 bp DNA fragment was amplified from either strain by using susceptible primers. The results revealed that both HD112^r and HD133^r had a Glu^{185} gene allele as is present in strain HD198^r instead of an Asp^{185} allele as is present in RC688^s.

The nucleotide substitution T→C at position 2288, which resulted in an amino acid replacement of IIe^{763} in RC688^s with Thr^{763} in the HD198^r strain, was also verified by using PASA amplification of genomic DNA extracted from individual larvae from both *P. interpunctella* strains. A single ~900 bp fragment (predicted to contain ~500 bp of introns) was amplified from individual larval DNA of RC688^s using a forward primer located 400 bp upstream of the point mutation on cDNA and a reverse primer corresponding to a susceptible allele (data not shown). No fragment was amplified from HD198^r strain when using the same primers. Thus, cDNAs of aminopeptidase-like protein genes from the Bt-susceptible and resistant *P. interpunctella* have only four nucleotide differences and encode proteins that differ in only two amino acid residues. The function of these encoded aminopeptidases is unknown, but they may play a role in peptide processing and degradation and/or serve as receptors for proteins such as Bt toxins.

Mohammed et al., 1996 reported that an 80 kDa protein bound to Cry1Ac, but it was unclear whether this protein was aminopeptidase-like. Other proteins, with cadherin- or alkaline phosphatase-like properties, have been proposed as Bt-toxin binding proteins (reviewed in Schnepf et al., 1998). However, aminopeptidase-like proteins act as receptors for Bt toxins in many insect species and are the most likely candidates for Bt toxin receptors in *P. interpunctella*. Our data provides the first evidence of sequence differences in a potential Bt toxin receptor in resistant insects, which could lead to altered toxin affinity.

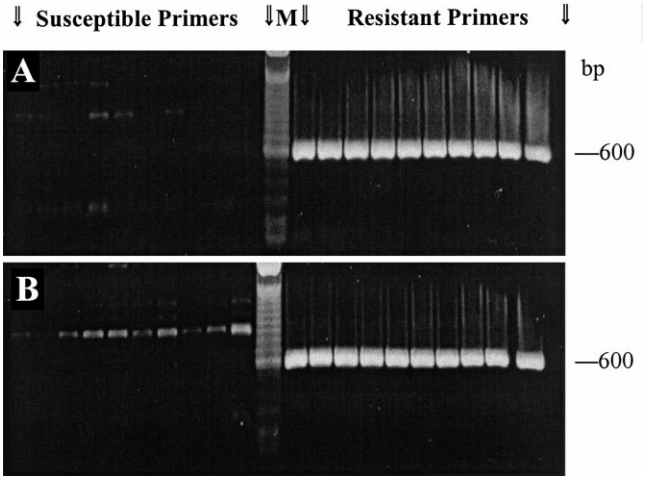

Fig. 5. PASA amplifications of individual larval DNA of Bt resistant HD112^r strain (A) and HD133^r strain (B). Ten individuals from each strain were used for each pair of primers. M=100 bp DNA ladder from Gibco Life-Technologies.

The significance of these results are two fold. Analysis of a putative Bt toxin receptor in Bt-susceptible and -resistant insects indicated that there is a conservation of a point mutation in resistant insects (Asp^{185}→ Glu^{185}) when compared to susceptible insects. More importantly, this mutation is localized to a region that is highly conserved among insects and is proposed to be a binding domain for Cry1Aa toxins. Therefore, alterations in this region could affect toxin binding, which could contribute to resistance. Alternatively, the second point mutation, resulting in a change from isoleucine^{763} in the susceptible insects to threonine^{763} in the resistant insects, is a change from a hydrophobic residue to one containing a potential site for a post translational modification, i.e., phosphorylation. This change could result in a substantial impact on the overall conformation of the molecule. Another possibility is that both mutations may have a combined effect that reduces toxin binding. The possibility of post-transcriptional or post-translational modifications of the receptor has not been ruled out. In some insects, the Cry1Ac toxin interacts with the carbohydrate moieties on the aminopeptidases (Lorence et al., 1997). Further studies are needed to determine the significance of these molecular differences in Bt-resistance development and to understand the mode of detoxification by insects resistant to Bt.

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